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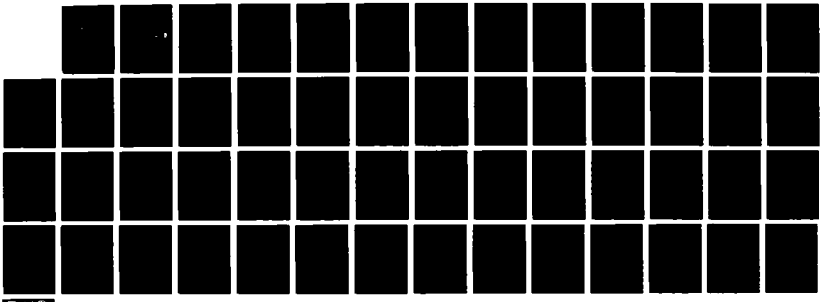

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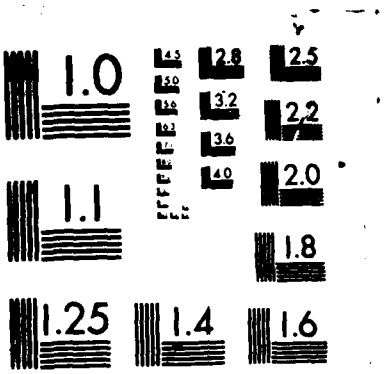

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PROTECTION AGAINST THE ACUTE AND DELAYED TOXICITY OF MUSTARDS AND MUSTARD-LIKE COMPOUNDS

ANNUAL/FINAL REPORT

DAVID B. LUDLUM

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Many of the toxicities of the sulfur mustards are caused by damage which these agents inflict on cellular DNA. This study was undertaken because previous data on DNA modifications caused by these agents did not seem adequate to explain all of their biological effects.

We have shown that a model sulfur mustard, chloroethyl ethyl sulfide (CEES), modifies the 6 position of guanine in DNA, producing O^6-ethylthioethyl deoxyguanosine. Although this lesion amounts to only 0.1% of the total DNA alkylation, its presence helps to explain the mutagenic effects of this agent. We have confirmed that the 7 position of guanine and the 3 position of adenine are the major sites of DNA modification; approximately 16% of the products of reaction between DNA and CEES have not been identified.
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19. Abstract (continued)

Formation of O^6-ethylthioethyl deoxyguanosine could be cytotoxic as well as mutagenic if the structure rearranged to produce an interstrand cross-link. Our data indicate that a cross-linking reaction does occur in DNA exposed to CEES.

The DNA repair enzyme, O^6-alkylguanine transferase, has been partially purified from rat liver nuclei. This enzyme removes methyl groups from the 6 position of guanine, but does not appear to have any activity toward the sulfur mustard-induced modification, O^6-ethylthioethyl deoxyguanosine.

Literature data indicate that other repair factors would be effective in repairing this DNA modification. Furthermore, we have obtained preliminary evidence that 7-ethylthioethyl deoxyguanosine is repaired by a factor in rat liver. Thus, it appears that mammalian cells have some ability to repair the DNA modifications caused by sulfur mustards. This suggests that resistance to the toxic effects of these agents can be enhanced.

An incidental finding in these studies is that CEES polymerizes. This reaction completely inactivates a single armed mustard and would partially inactivate a bifunctional mustard.

Keywords: → contd pg A

SUMMARY

The sulfur mustards cause serious toxicities to the skin, respiratory tract, bone marrow, and sometimes to other organs as well; carcinogenesis may appear as a final toxicity many years after exposure. Probably all of these effects, but certainly the bone marrow depression and carcinogenesis, are related to the damage caused to cellular DNA by these agents (1,2). These investigations were undertaken because the data which were available previously on the nature of the DNA modifications caused by these agents did not seem adequate to explain all of their biological effects.

Genetic studies with monofunctional sulfur mustards suggest that alkylation of the 6 position of guanine in DNA is responsible for some of the mutagenic effects of these agents; similar lesions would, of course, be caused by bifunctional sulfur mustards (2). The studies described in this report address the following questions: a) Does a typical sulfur mustard like chloroethyl ethyl sulfide (CEES) attack the 6 position of guanine and b) is this lesion repaired by the enzyme, O^6-alkyl guanine-DNA alkyl transferase (alkyl transferase).

We have shown by chemical methods that CEES does modify the 6 position of guanine in DNA, producing a small amount of O^6-ethylthioethyl deoxyguanosine. Although this lesion amounts to only 0.1% of the total DNA alkylation, it could have considerable biological significance. At the same time, we have confirmed that the major sites of DNA modification are the 7 position of guanine and the 3 position of adenine. Approximately 16% of the DNA modification is at still unknown sites.

Formation of O^6-ethylthioethyl deoxyguanosine is probably carcinogenic but this DNA modification could be cytotoxic as well if the structure rearranged, as do similar modifications caused by antitumor agents, to produce an interstrand cross-link. Chromatographic data have been obtained which indicate that this cross-linking reaction may occur in DNA exposed to CEES.

The DNA repair enzyme, O^6-alkylguanine transferase, has been isolated from rat liver nuclei and partially purified by ammonium sulfate fractionation and DNA cellulose chromatography. This enzyme, although active in the removal of methyl groups from the 6 position of guanine, does not appear to have any activity toward the sulfur mustard-induced modification, O^6-ethylthioethyl deoxyguanosine.

Literature data do indicate, however, that other repair factors would be effective in repairing this DNA modification. Furthermore, we have obtained preliminary evidence that 7-ethylthioethyl deoxyguanosine is repaired by some factor in rat liver. Thus, it appears that mammalian cells do have some ability to repair the DNA modifications caused by sulfur mustards. This, in turn, offers hope that resistance to the toxic effects of these agents can be enhanced.

An incidental finding in these studies is that CEES polymerizes. This reaction completely inactivates a single armed mustard and would partially inactivate a bifunctional mustard.

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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INTRODUCTION

The sulfur mustards cause several important toxicities which appear in a definite time sequence after exposure. Toxicities to the skin and the respiratory tract appear almost immediately. Toxicity to the bone marrow becomes apparent after a few days, often with serious medical consequence. Finally, carcinogenesis may appear many years later. Probably all of these toxicities, but certainly the bone marrow depression and carcinogenesis, are related to the damage caused to cellular DNA by these agents (1,2).

When these investigations were initiated, chemical evidence had been obtained that sulfur mustards attack the N-7 position of guanine and the N-3 position of adenine in DNA (3-5). Furthermore, DNA cross-linking had been demonstrated through attack on the N-7 positions of neighboring guanines (6). Studies in repair-deficient strains of bacteria by Gibert et al. (7) indicated, however, that other lesions were important in producing the mutagenic and carcinogenic (i.e., the late) toxicities of the sulfur mustards. A likely cause of these toxicities appeared to be alkylation of the O^6-position of guanine in DNA, but this modification had not actually been demonstrated when we began the investigations supported by this contract.

Since it has become clear that minor modifications of DNA by environmental agents can be more important than modification at the major alkylation sites, an important initial objective of this work was to identify the minor products of DNA alkylation by the sulfur mustards, with special emphasis on the O^6-position of guanine.

The studies of Gilbert et al. (7) also indicated that the postulated O^6-alkyl guanine lesion was repaired by some unknown cellular mechanism. Several groups including our own (8-11) had shown that both bacterial and mammalian cells contain a repair factor which removes alkyl groups from the O^6-position of guanine and attaches them to a cysteine moiety within the repair protein itself. It seemed possible that this same repair mechanism would remove sulfur mustard-derived alkyl groups from the O^6-position of guanine and afford protection to the toxic effects of the sulfur mustards. Consequently, a second important objective was to obtain further information on this enzyme in mammalian cells and to determine whether it would repair sulfur mustard-induced alkylation of the O^6-position of guanine. At the same time, we would be alert for other mechanisms which might repair sulfur mustard-induced damage to DNA.

As an additional objective, added as an extension to the original contract, we have investigated some of the chemical consequences of the alkylation of the O^6-position of guanine in DNA. A mechanism has been discovered for certain antitumor agents which leads to DNA cross-linking following alkylation of the O^6-position of guanine as a first step (12). If such a mechanism occurs with the sulfur mustard, and our studies indicate that it may, this would attach further significance to any mechanism which can repair this lesion.

DNA modification and repair has been an active area for research in the past few years, and the current state of knowledge is summarized in the Background section which follows. Our methods of investigating DNA modification

by the sulfur mustards and repair of these lesions are then described, and the results which we have obtained over the past 3 1/2 years are presented in a unified fashion. Finally, the significance of these results is discussed, and recommendations are made for their application.

BACKGROUND

DNA Modification by the Sulfur Mustards

Early studies of DNA modification by the sulfur mustards were performed before high pressure liquid chromatography (HPLC) methods were available to detect and characterize minor DNA modifications (13). Nevertheless, the major sites of alkylation, the N-7 position of guanine and the N-3 position of adenine, were identified. Also, the importance of cross-linking between the N-7 positions of neighboring guanines was fully documented (6).

Studies carried out at the USAMRDC indicated, however, that other DNA modifications might also be important (7). Furthermore, recent investigations of the mode of action of antitumor agents and of environmental carcinogens have emphasized the importance of DNA modifications at other sites besides the N-7 position of guanine and the N-3 position of adenine (14). Indeed, Singer and Grunberger (15) have shown that all of the nitrogens and oxygens in DNA can react with alkylating carcinogens.

Although most cross-links which are formed by the sulfur mustards are between neighboring guanines in DNA, other mechanisms of interstrand and intrastrand cross-link formation have been discovered. Simple monofunctional alkylating agents, including methylating agents, appear to cause cross-linking through the reactions of the free sugars left at depurination sites (16). Haloethylnitrosoureas and perhaps other chloroethylating agents form cross-links between the N-1 position of guanine and the N-3 position of cytosine following an initial attack of the chloroethyl group on the O^6-position of guanine (12). Both kinds of cross-links are probably important, but the second one is definitely known to be cytotoxic (17). Furthermore, recent data have indicated that mammalian cells can develop resistance to this lesion (17).

Thus, data from a variety of sources indicate that DNA modification by alkylating agents is more complex than previously appreciated and that some of the minor DNA modifications have important biological consequences.

Repair of Sulfur Mustard-induced Lesions in DNA

Early investigations of DNA repair in bacteria centered on the role of AP endonuclease in repairing apurinic sites. The chemical lability of the bond between deoxyribose and 7-alkyl guanine or 3-alkyl adenine had been recognized, and the spontaneous release of these bases had been observed. The resulting apurinic sites were shown to be recognized by an AP endonuclease which incises the sugar phosphate backbone. The gap is enlarged by an exonuclease and then repaired by the subsequent action of DNA polymerase and DNA ligase.

More recently, a group of enzymes has been recognized in bacteria which catalyze the release of modified DNA bases (18). Two separate 3-methyl adenine DNA glycosylases have been described in Escherichia coli, 3-methyl adenine DNA glycosylase I and 3-methyl adenine DNA glycosylase II (19). The first of these enzymes has no activity toward 7-methyl guanine, and the second has only weak activity toward this lesion (19). Thus, E. coli evidently does not contain a glycosylase which would actively repair the most prevalent site of sulfur mustard-induced DNA alkylation.

Glycosylases from mammalian cells may be more active toward 7-alkyl guanines, however. A glycosylase that has activity toward 7-methyl guanine has recently been isolated from rat liver by Margison and Pegg (20) and from calf thymus by Male et al. (21). The suggestion has been made that glycosylases of bacterial origin recognized modified bases which protrude into the minor groove, while mammalian glycosylases recognized modifications which are positively charged (18). In any case, the papers by Margison and Pegg (20) and by Male et al. (21) provide support for our data which indicate that 7-ethylthioethyl guanine is enzymatically released by rat liver extract.

In addition to glycosylases, other pathways evidently assist in the repair of sulfur mustard-induced lesions. Bacteria which have lost the UVR excision endonuclease are especially sensitive to the sulfur mustards, as are bacteria deficient in rec-A-dependent repair (7). Many questions remain, however, as to which lesions are recognized by these various repair mechanisms.

When we began these investigations, it seemed likely that the newly discovered O^6-alkyl guanine-DNA alkyl transferase (alkyl transferase) would repair O^6-alkylation by sulfur mustards. However, our data, which are reported below, indicate that O^6-alkyl transferase from rat liver is unable to repair O^6-ethylthioethyl guanine. Recently published studies corroborate this finding; Morimoto et al. (22) have shown that both E. coli and rat liver alkyl transferase show less activity for larger, bulkier alkyl groups than they do for methyl and ethyl groups. Larger, bulkier DNA modifications are, however, exactly the kinds of lesions which are repaired by the UVR excision endonuclease system in bacteria, and it is probable that a similar system would provide protection from such modifications in mammalian cells. Since the studies of Gilbert et al. (7) suggest that O^6-ethylthioethyl guanine is repaired, we might conclude that the UVR excision endonuclease recognizes this lesion and assists in its repair.

Thus, at the same time that new DNA are being recognized, additional information is becoming available on the cellular repair mechanisms which provide protection against them. In this regard, it should be emphasized that alkylating carcinogens are normal constituents of the environment and that it can be anticipated that cells would have defenses against alkylation. That these defenses can be enhanced is suggested by the fact that tumor cells readily become resistant to the alkylating activity of DNA-modifying antitumor agents (23).

MATERIALS AND METHODS

Materials

Chloroethyl Ethyl Sulfide

Chloroethyl ethyl sulfide (CEES) was obtained from two independent commercial sources: Chemical Procurement Labs, Inc., College Point, NY, and Parish Chemicals, Orem, UT. Some of this material was stored undiluted at freezer temperatures (-20°C) and some at room temperature. It was discovered that CEES polymerizes at both temperatures to a form which is no longer reactive with deoxynucleosides. This phenomenon was investigated by gas chromatography and mass spectrometry and is fully documented in a previous report and summarized in the appendix (24). Fresh CEES was used in all of the experiments described below.

[^{14}C]-Chloroethyl Ethyl Sulfide

[^{14}C]-Chloroethyl-labeled chloroethyl ethyl sulfide ([^{14}C]-CEES) was custom synthesized by Amersham as described previously (24). It had a specific activity of 27 mCi/mmol or 56.4 dpm/pmol when counted in our Beckman LS-1800 scintillation counter.

[^{14}C]-CEES was stored as a very dilute toluene solution at freezer temperatures (-20°C) and remained stable, probably because dilution in toluene prevents the bimolecular polymerization reactions mentioned above.

Other Reagents

Hemisulfur mustard was synthesized by the method of Tsou et al. (25) as described in our first Annual Report (26). Deoxynucleosides were obtained from P-L Biochemicals. Calf thymus DNA and enzymes for its digestion came from Cooper Biomedical, Inc. (Cappel/Worthington Scientific Division, Malvern, PA). Deoxycoformycin, an adenosine deaminase inhibitor, was supplied by the Division of Cancer Treatment, National Cancer Institute. O^6-Ethylthioethyl deoxyguanosine, used as a marker in HPLC analyses, was prepared as described previously (26,27). 7-Ethylthioethyl deoxyguanosine, 7-ethylthioethyl guanine, 3-ethylthioethyl adenine, and 3-ethylthioethyl deoxycytidine, which were also used as HPLC markers, were prepared as described in the Results section below. [^{3}H]-Methylnitrosourea (1.2-1.6 Ci/mmol), used for preparing ^{3}H-methylated DNA as a substrate for assaying alkyl transferase activity, was obtained from New England Nuclear (Boston, MA). The unlabeled HPLC marker, O^6-methyl guanine, used in this assay was synthesized in our laboratory from 6-chloro-2-amino-purine and sodium methoxide. Ethidium bromide was obtained from Calbiochem (San Diego, CA). All other reagents came from standard sources.

Methods

Reactions of CEES with Deoxynucleosides and Bases

To investigate DNA modification and repair by the sulfur mustards at realistic levels of DNA damage, it is necessary to use radiolabeled CEES to modify the DNA. With ^{14}C-labeled CEES at a specific activity of 27 mCi/mmol, picomole levels of modified nucleosides or bases can be detected by their appearance as radioactive peaks in an HPLC separation. At these levels, however, it is not possible to obtain any chemical information to verify the identity of the radiolabeled peaks. Accordingly, it is necessary to prepare and characterize modified deoxynucleosides and bases so that the identity of the radiolabeled peak can be verified by co-chromatography with known marker compounds.

We have previously described the synthesis of O^6-ethylthioethyl deoxyguanosine from 6-chlorodeoxyguanosine (26,27). O^6-Hydroxyethylthioethyl deoxyguanosine was synthesized by an entirely analogous route (28). Subsequently, we have found that marker amounts of O^6-ethylthioethyl deoxyguanosine can be prepared by the direct reaction of CEES with deoxyguanosine in dimethyl sulfoxide (DMSO) in the presence of potassium carbonate as described in the Results section.

Marker amounts of 7-ethylthioethyl deoxyguanosine, 7-ethylthioethyl guanine, 3-ethylthioethyl adenine, and 3-ethylthioethyl deoxycytidine have all been prepared by reacting the corresponding deoxynucleosides or bases at 1 mg/ml in 50 mM sodium cacodylate buffer, pH 7, with chloroethyl ethyl sulfide at a concentration of 1-4 mg/ml; pH was maintained at 7 in these reactions by the addition of small amounts of 1 N NaOH. The complex reaction mixtures obtained in this way were separated by reverse-phase chromatography on a C_{18} column as described in the Results section. Purified derivatives were characterized by ultraviolet and mass spectrometry and identified by comparison with literature data.

Isolation of 1-(3-Deoxycytidyl),2-(1-deoxyguanosinyl)ethane

This DNA crosslink was isolated from DNA treated with N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU) following the procedure described by Tong et al. (12). Briefly, 500 mg of calf thymus DNA was dissolved in 62 ml of 25 mM sodium cacodylate buffer, pH 7. BCNU (250 mg) in 1 ml of 95% ethanol was added, and the solution was incubated overnight at 37°C. At the end of this period, 62 ml of the same buffer, 1 ml of 1 M $MgCl_2$, and 1.2 ml of 6 M NaCl were added together with 40 units of snake venom phosphodiesterase, 4 units of spleen phosphodiesterase, 2000 units of DNase I, and 20 units of bacterial alkaline phosphatase. Six hr later and again at 24 hr, 20 units of venom phosphodiesterase, 2 units of spleen phosphodiesterase, 1000 units of DNase I, and 10 units of bacterial alkaline phosphatase were added.

After 48 hr, this digest was applied to an SP-Sephadex C-25 column (2.5 x 60 cm) and eluted at 1 ml/min with a linear gradient of sodium formate, pH 6, 0.05 M to 0.5 M (total volume, 1600 ml); under these conditions, most modified nucleosides and dinucleosides are positively charged and are

retained by this resin; unmodified nucleosides elute in the front. Fractions 69-84 contained about 10 A_{254} units which had the 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane structure described previously (12).

Attempts to synthesize 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)-ethane using deoxycytosine and deoxyguanosine as starting materials have not been successful. Apparently, the secondary structure of DNA actually facilitates the formation of this cross-linked entity, and synthesis from its constituent deoxynucleosides will probably be very difficult.

Both glycoside bonds in this structure are labile to acid so that the corresponding 1-(3-cytosinyl),2-(1-guanyl)ethane can be released from DNA which contains the cross-link. Marker amounts of 1-(3-cytosinyl),2-(1-guanyl)-ethane were prepared from 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane by treatment with 0.1 N HCl at 100°C for 60 min. Larger amounts of this derivative were prepared by the direct synthesis described below.

Synthesis of 1-(3-Cytosinyl),2-(1-guanyl)ethane

The mechanism proposed for DNA cross-linking by the haloethylnitrosoureas is a sequential one in which a haloethyl group is first attached to the O^6-position of guanine and then rearranges to form an ethano bridge between the 1- and O^6-positions. The resulting intermediate, 1,O^6-ethanoguanine, is evidently the reactive species which forms a cross-link with the cytosine in the opposite DNA strand. This postulated sequence of reactions suggested a mechanism for synthesizing 1-(3-cytosinyl),2-(1-guanyl)ethane from monomeric materials.

O^6-Fluoroethylguanosine can be readily synthesized from commercially available starting materials as described in the literature (29). According to the mechanism proposed above, this compound should react with deoxycytidine to form 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane. Acid hydrolysis of this derivative should lead to 1-(3-cytosinyl),2-(1-guanyl)-ethane, the same product which is liberated from 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane by acid.

This synthesis was, in fact, successful. O^6-Fluoroethylguanosine was first prepared as follows: Sodium hydride (80 mg) was added to 2-fluoroethanol (2 ml) contained in a 10-ml round bottomed flask at room temperature. After a few minutes, when the reaction of sodium hydride with 2-fluoroethanol was complete, 6-chloro-2-amino-purine-9-riboside (50 mg) was added and the solution was stirred at room temperature for 5 min and then at 55°C for 50 min. Unreacted 2-fluoroethanol was removed under vacuum and the residue was dissolved in 5 ml of water. This solution was adjusted to neutral pH with dilute HCl, filtered, and separated on a G-10 column (2 x 100 cm) which was eluted with water at a flow rate of 1 ml/min. Ten milliliter fractions were collected and fractions 77-94 were found to contain O^6-fluoroethylguanosine as described previously (29). These fractions were pooled and lyophilyzed to dryness, and the solid O^6-fluoroethylguanosine was stored over silica gel.

This compound was then reacted with deoxycytidine in DMSO as follows: O^6-fluoroethylguanosine (4 mg) was added to 80 µl of dimethylsulf-

oxide containing 6.4 mg of deoxycytidine. This mixture was incubated at 55°C for 2 weeks. At the end of this time, it was added to 8 ml of water and separated, 1 ml at a time, on a C_{18} column eluted at 1 ml/min with a 1-10% gradient of acetonitrile in 50 mM KH_2PO_4, pH 6, over a 20-min period and then with 10% acetonitrile in the same buffer for an additional 20 min. The absorbance of the eluent was monitored at 280 nm, and each major peak was collected and compared with known derivatives. As described in the Results section, one of these peaks was shown to have the structure, 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane.

Reactions of [^{14}C]-CEES with DNA

DNA was reacted with [^{14}C]-CEES at several [^{14}C]-CEES concentrations in order to obtain a suitably modified DNA for repair studies. Calf thymus DNA was dissolved at a concentration of 16 mg/ml in 50 mM sodium cacodylate buffer, pH 7.3, by rotating the solution slowly at 37°C for 24 hr. Then an equal volume of 95% ethanol was added and the rotation was continued until the solution appeared homogeneous. The resulting solution was viscous, but clear, and the limited amount of water present increased the yield of DNA alkylation by the [^{14}C]-CEES. Presumably, the presence of ethanol not only facilitates transfer of the [^{14}C]-CEES from the toluene solution, but also reduces the concentration of water which inactivates the [^{14}C]-CEES. The use of cacodylate buffer prevented bacterial growth during the slow dissolution process.

[^{14}C]-CEES, 1-30 µg/ml, was added to this solution and allowed to react with the DNA for 3-4 hr. Then, the solution was made 0.3 M in NaCl and 2 additional volumes of 95% ethanol were added. The DNA was collected by centrifugation, redissolved at a concentration of approximately 8 mg/ml, and reprecipitated with ethanol several times until the amount of radioactivity in the supernatant had been reduced to a constant level. As noted in the Discussion section, there is a continued slow release of radioactivity from DNA, presumably because of spontaneous loss of alkylated bases and, perhaps, alkyl groups. After the last precipitation, the DNA was redissolved in buffer A (50 mM Tris-HCl, pH 7.8, containing 1 mM Na_2EDTA, 5% glycerol, and 5 mM dithiothreitol) at approximately 2 mg/ml, divided up into multiple aliquots, and stored frozen at -20°C until needed for repair studies.

Digestion of [^{14}C]-CEES-modified DNA

[^{14}C]-CEES-modified DNA was dissolved in buffer A at a concentration of 1 mg/ml or less and digested by adding the following per milliliter of solution:

Reagent	Amount
1 M NaCl	4 µl
1 M $MgCl_2$	10 µl
Bacterial alkaline phosphatase	1 unit
Spleen phosphodiesterase	0.2 unit
Venom phosphodiesterase	1 unit
Deoxyribonuclease I	100 units
Deoxycoformycin	10 µg

An overnight incubation at 37°C was found to be sufficient for complete digestion. After digestion and prior to HPLC analysis, the solutions were filtered through an 0.2-μ membrane filter, and an aliquot of the filtrate was counted in 3.5 ml of Hydrofluor to determine the amount of radioactivity in the total filtrate.

In order to provide a comparison with published data on the reaction of CEES with DNA, modified bases have also been released from [^{14}C]-CEES-modified DNA by an acid depurination procedure. This method consists of heating the DNA in 0.1 N HCl at 70°C for 20 min, neutralizing and filtering the solution, and analyzing the modified bases released by this procedure by HPLC as described below.

HPLC Analysis of [^{14}C]-CEES-modified DNA Digest

High pressure liquid chromatographic analyses of [^{14}C]-CEES-modified DNA digests were performed on a modular apparatus consisting of a Milton-Roy 5000 psi minipump, a Rheodyne 7125 injector valve, an LDC Fluoromonitor III fluorometric detector, and a Hewlett-Packard 1040A detector system. This detector is interfaced with a Hewlett-Packard 85 computer with disc drive and is able to record spectra of derivative peaks as they are separated. Eluent fractions were collected automatically in an ISCO fraction collector.

Filtered DNA digests containing [^{14}C]-CEES-modified deoxynucleosides and bases were separated on a 5-μ C_{18} reverse-phase column (4.6 x 250 mm), which was eluted isocratically at 1 ml/min with 14% acetonitrile in 25 mM KH_2PO_4, pH 4.5. This system separated the late appearing radioactive peak of O^6-ethylthioethyl deoxyguanosine from all other peaks of radioactivity. Fractions of eluent were collected in minivials every 0.5 min, 3.5 ml of Hydrofluor was added to each vial, and fractions were counted for 5 min in an LS-1800 Beckman scintillation counter.

Profiles of radioactivity versus elution time were then plotted on a Hewlett-Packard 85 computer, and the amount of radioactivity in each radioactive peak in the profile was determined with a computer program which automatically subtracts the background. These profiles were highly reproducible and recovery of injected radioactivity averaged 100%. Seven distinct peaks of radioactivity were observed; most of these have been identified as described in the Results section below.

Isolation of Alkyl Transferase

Alkyl transferase was isolated from the livers of male Sprague-Dawley rats (150-175 g) which were housed in our animal quarters and which were allowed access to food and water at all times. A two-thirds partial hepatectomy was carried out under ether anesthesia 2 days before sacrifice to increase the yield of repair protein. The livers, typically 90 g from 15 rats, were homogenized in 3 volumes of buffer A (50 mM Tris-HCl, pH 7.8, containing 1 mM Na_2EDTA, 5% glycerol, and 5 mM dithiothreitol) containing 0.5 mM concentration of the proteinase inhibitor, PMSF; this homogenization and all subsequent steps in the purification were performed at 4°C. The homogenate was centrifuged at 12,000 g for 10 min, and the supernatant was

removed and saved. An additional 1 volume of buffer A containing PMSF was added to the pellets, and the suspension was sonicated for three periods of 30 sec, each at 1-min intervals. This extract was added to the previously collected supernatant, mixed, and centrifuged at 22,000 g for 30 min. The supernatant from this step is described as the initial extract in Table 1.

Solid ammonium sulfate was then added to bring the solution to 25% saturation. After stirring for 30 min, the precipitate was removed by centrifugation at 22,000 g for 10 min. The resulting supernatant was adjusted to 55% saturation by adding additional solid ammonium sulfate. After stirring for 30 min, the precipitate was collected by centrifugation at 22,000 g for 10 min and redissolved in a minimum volume of buffer A. This solution was dialyzed overnight against buffer A, centrifuged at 22,000 g for 30 min to remove insoluble material, and the supernatant saved as fraction II (25-55% ammonium sulfate cut) in Table 1.

Fraction II was then purified 50 ml at a time through DNA cellulose columns (2.5 x 12 cm) prepared by the method of Alberts and Herrick (30) and pre-equilibrated with buffer A. The column was eluted with buffer A at a flow rate of 0.2 ml/min until the A_{280} was 0 and then with buffer A containing 50 mM NaCl, again until the A_{280} was 0. Repair activity was then eluted with buffer A containing 0.25 M NaCl. Fractions of 4 ml were collected every 20 min, and those with A_{280} greater than 0.04 were pooled and stored as an ammonium precipitate at -70°C (DNA cellulose fraction).

Typical results from such a purification are given in Table 1. In this table, one unit of enzyme activity corresponds to the removal of 1 pmol of [^{3}H]-methyl groups from O^6-methyl guanine in DNA which has been alkylated with [^{3}H]-methylnitrosourea (MNU) as described below.

Table 1

Purification of Alkyl Transferase

Step	Total Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification Factor (-fold)
Initial extract	37,600	0.04	---	---
25-55% Ammonium sulfate cut, postdialysis	11,100	0.05	39	1.3
DNA cellulose	2.5	22.0	4	550

As is often the case with mammalian enzymes, the enzyme becomes less stable as greater purification is achieved. Fractions from the DNA cellulose column can, however, be stored at -70°C without loss of activity as ammonium sulfate precipitates. When this enzyme is redissolved in buffer A, it can be stored without serious loss of activity for several days at -70°C.

Assay of Alkyl Transferase Activity

One unit of transferase activity is defined as that amount of activity which will remove 1 pmol of methyl groups from O^6-methyl guanine in methylated DNA. In calculating specific activity, protein concentrations were determined by the dye-binding method of Bradford (31) using bovine serum albumin as a standard.

DNA methylated with [^{3}H]-methylnitrosourea was used as substrate in this assay. This substrate was prepared by reacting calf thymus DNA at a concentration of 3 mg/ml in 0.2 M Tris-HCl, pH 8, with 150 mCi/ml of [^{3}H]-methylnitrosourea (1.6 Ci/mmol) at 37°C.

After 2 hr, the alkylated DNA was precipitated by the addition of 0.1 volume of cold 2 M sodium acetate, pH 5, and 2 volumes of cold 95% ethanol. The precipitated DNA was collected by centrifugation and washed free of unbound radioactivity by repeated dissolution and reprecipitation. The pellet was air dried and redissolved in 3 ml of 50 mM Tris-HCl, pH 7.8, which contained 0.1 mM Na_2EDTA.

Purified enzyme fractions were assayed in a total volume of 0.25 ml of buffer A in Eppendorf centrifuge tubes. [^{3}H]-Methyl DNA containing approximately 0.6 pmol of O^6-methyl guanine was added and the assay tubes were incubated for 1 hr at 37°C. Then, 50 µg of unlabeled DNA was added as carrier, and the DNA was precipitated by the addition of 0.1 volume of cold 2 M sodium acetate, pH 5, and 2 volumes of cold ethanol. After 2 hr at -20°C, the DNA was collected by centrifugation for 5 min at full speed in the Eppendorf microfuge. The supernatant was removed and discarded, and the pellet was resuspended in 0.95 ml of 0.1 N HCl and depurinated for 30 min at 70°C. The solution was then cooled in an ice bath, neutralized with 2 N Na_4OH, and the supernatant separated by centrifugation.

The purines present in each supernatant were separated by HPLC on a Spherisorb ODS column (5 µ, 4.0 x 250 mm) eluted at a flow rate of 1 ml/min with a 20-min linear gradient of 3-10% acetonitrile in 25 mM KH_2PO_4, pH 3.5, followed by 10% acetonitrile in the same buffer. In this system, O^6-methyl guanine eluted at 22.5 min, well after the other methylated purines. One-half-minute fractions were collected, dissolved in 3.3 ml of Hydrofluor, and counted in an LS-1800 Beckman scintillation counter. The number of picomoles of methyl groups removed from O^6-methyl guanine in the DNA was calculated from a comparison with control tubes, making the appropriate corrections for quenching, and this number was used to determine the alkyl transferase activity.

Effect of Alkyl Transferase on [^{14}C]-CEES-modified DNA

Since alkyl transferase repairs O^6-alkyl guanines in DNA by removing the alkyl group, this repair activity can be detected by monitoring the disappearance of ^{14}C-labeled groups from O^6-ethylthioethyl guanine in DNA which has been modified with [^{14}C]-CEES as described above. This assay scheme is shown in Figure 1. As this figure indicates, it is important to perform parallel incubations of substrate DNA with active and inactive protein and to add O^6-ethylthioethyl deoxyguanosine marker to the DNA digests before performing the HPLC analyses. This is because the small amount of ^{14}C-labeled O^6-ethylthioethyl deoxyguanosine contained in the substrate DNA may be artifactually lost by binding to the protein or other surfaces unless it is displaced by the unlabeled marker.

Typical incubation mixtures for this assay contained 0.26 mg of [^{14}C]-CEES-modified DNA bearing 1.6 pmol of ^{14}C-labeled O^6-ethylthioethyl deoxyguanosine and varying amounts of repair enzyme in 0.7 ml of buffer A. Substrate DNA and repair enzyme were incubated together for 1 hr at 37°C, 6 nmol of marker O^6-ethylthioethyl deoxyguanosine was added, and the DNA was digested in the same incubation tube using the method described above. After the digestion was complete, the solution was filtered through a 2-μ membrane filter, and an aliquot of the filtrate was counted in 3.5 ml of Hydrofluor to determine the total amount of radioactivity in the filtrate. A known volume of filtrate was then analyzed by HPLC as described above. For purposes of comparing experimental and control runs at different levels of enzyme concentration, the amount of radioactivity in each peak was converted to picomoles of derivative per mg of DNA.

Use of Doubly-Labeled [^{3}]H-Thymidine [^{14}C]-CEES-modified DNA to Detect Glycosylase Activity

The standard assay for glycosylase activity consists of measuring the release of radiolabeled bases from substrate DNA prepared by reacting a suitable DNA with radiolabeled alkylating agent. After exposure to glycosylase activity, the substrate DNA is precipitated with alcohol and the supernatant is examined for the presence of radioactivity.

However, most partially purified DNA gylcosylases contain some endonuclease activity. This activity can create artifactual counts in the supernatant by degrading DNA through scission of the sugar phosphate backbone. If the substrate DNA contains randomly distributed [^{3}H]-thymidine in addition to [^{14}C]-CEES-modified bases, however, specific release of the ^{14}C-labeled bases can be detected by the appearance of ^{14}C-labeled material in the supernatant at a higher ^{14}C/^{3}H ratio than in the substrate DNA.

Such a substrate was prepared by using the method of "nick-translation" to incorporate [^{3}H]-thymidine into calf thymus DNA and then alkylating this substrate with [^{14}C]-CEES. Nick translation was performed using a nick translation kit obtained from Cooper Biomedical, Inc., Malvern, PA. The reaction mixture consisted of the following:

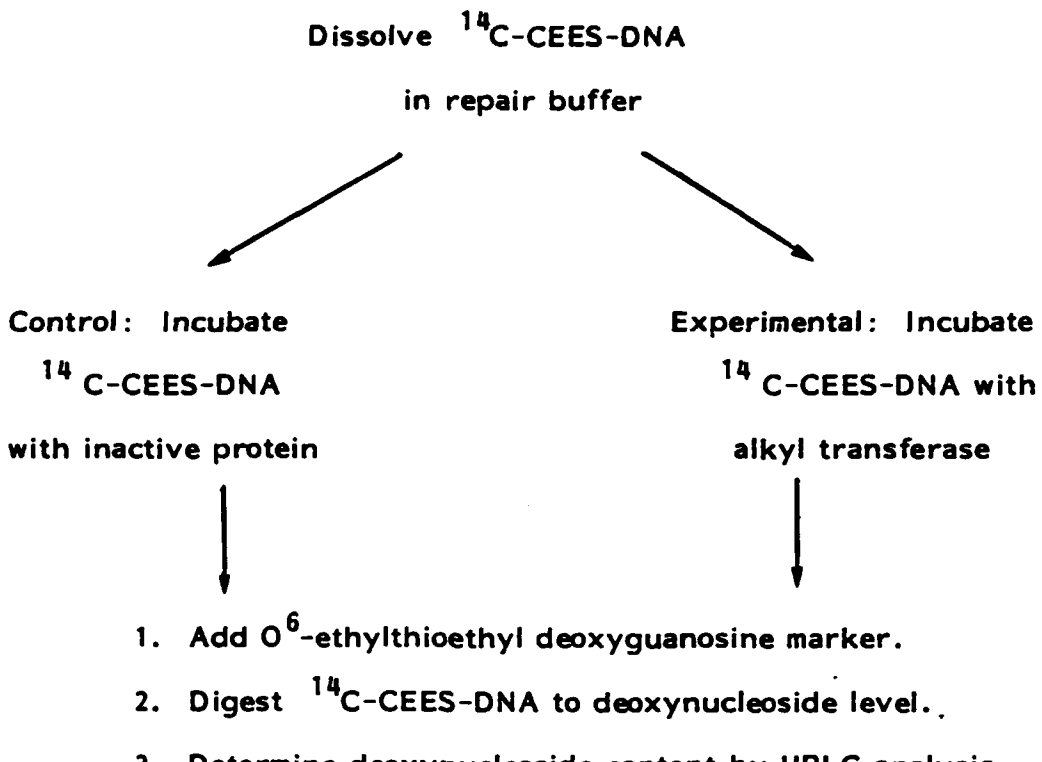

Figure 1. Scheme for investigating repair of O^6-ethylthioethyl guanine in DNA by alkyl transferase.

Total volume	50 µl
Buffer*	50 mM
dATP	20 µM
dGTP	20 µM
dCTP	20 µM
dTTP	8 µM, 1 µCi
DNA polymerase	5 units
DNA	0.3 µg

*50 mM KH_2PO_4, pH 7.00, 0.1 mM EDTA, 500 µg/ml of BSA, and 50% glycerol.

This reaction mixture was incubated for 1 hr at 16°C and then polymerase action was terminated by adding 2 µl of 0.5 M EDTA and heating the solution to 70°C for 5 min. DNA was precipitated by adding 3 µl of 6 M NaCl and 200 µl of 95% ethanol. The DNA was then successively redissolved in 100 µl of 50 mM sodium cacodylate buffer, pH 7.1, containing 1% sodium pyrophosphate and precipitated with 5 µl of 6 N NaCl and 400 µl of 95% ethanol. After the fourth wash, no more radioactivity appeared in the supernatant; 240,000 cpm of [^{3}H]-thymidine remained in the DNA.

This DNA was mixed with 5 mg of unlabeled calf thymus DNA dissolved in 1.5 ml of 50 mM sodium cacodylate buffer, pH 7.1. The tube containing this mixture was rotated in a 37°C bath for 1 hr to ensure homogeneity and then [^{14}C]-CEES was added to a final concentration of 0.63 mM. Incubation was continued for 3 hr and the DNA was precipitated with ethanol and washed free of uncombined radioactivity by repeated cycles of the dissolution and reprecipitation.

The resulting DNA contained approximately 3000 cpm's of [^{3}H]-thymidine and 35,000 cpm's of [^{14}C]-CEES-modified bases per A_{260} unit.

Ethidium Bromide Fluorescence Method for Measuring DNA Cross-linking

DNA cross-linking was measured by the method of Morgan and his associates (32,33). This method depends on the fact that ethidium bromide intercalated in double helical DNA is much more fluorescent than when it is free in solution. Thus, the intensity of fluorescence can be used to monitor the amount of double helical DNA in solution. Uncross-linked DNA which has been subject to heat denaturation no longer intercalates ethidium bromide and gives a lower reading than cross-linked DNA which renatures and exhibits a higher fluorescence.

DNA for cross-linking studies was reacted with [^{14}C]-CEES in 50 mM sodium cacodylic buffer, pH 7.4, as described above; the extent of alkylation was determined by measuring the bound radioactivity per milligram of DNA and converting to molar amounts using the known specific activity of the [^{14}C]-CEES.

Alkaline heat denaturation was performed in 20 mM K_3PO_4, pH 11.8, containing 0.4 mM EDTA and 0.4 µg/ml of ethidium bromide by heating the solution in a boiling water bath for 3 min followed by cooling in an ice bucket. Three

millliliters of fluorescence buffer was used for each determination, and each tube contained 0.4 A_{260} unit of DNA.

Fluorescence was measured before and after heat denaturation in a Perkin-Elmer 512 fluorescence spectrophotometer using an excitation wavelength of 525 nm and measuring the emission at wavelengths greater than 600 nm. Fluorescence before heating varied less than 5%. The fully cross-linked positive controls which were prepared by reaction with nitrogen mustard regained approximately 20% of the original fluorescence after they were subjected to a denaturation cycle.

RESULTS

Synthesis of Modified Deoxynucleosides and Bases

Rationale

In order to study the modification and repair of sulfur mustard-induced DNA lesions at realistic levels of DNA modification, it is necessary to use radiolabeled sulfur mustards to modify DNA. Identification of the lesions introduced into substrate DNA by this procedure then depends entirely on co-chromatography of radiolabeled deoxynucleosides and bases released from the substrate DNA with known, well-characterized derivative.

Previous reports in this series (24,25,27) have included a description of the reaction of chloroethyl ethyl sulfide and of hemi-sulfur mustard with deoxynucleosides and bases at neutral pH. These reactions were performed to provide background on the kinds of modifications that would be caused by the reaction of CEES with DNA. The variety of derivatives that we found was somewhat unexpected in light of the literature emphasis on alkylation of deoxyguanosine on the 7 position and of deoxyadenosine on the 3 position.

Since our initial objective was to investigate alkylation of deoxyguanosine in the O^6-position by the sulfur mustards, unambiguous organic syntheses of O^6-ethylthioethyl deoxyguanosine, the product to be expected from CEES, and of O^6-hydroxyethylthioethyl deoxyguanosine, the product to be expected from hemi-sulfur mustard, were performed through the intermediate, 6-chloro deoxyguanosine (26-28). Subsequently, we demonstrated that O^6-ethylthioethyl deoxyguanosine was also formed when CEES reacted with deoxyguanosine or with DNA and that O^6-hydroxyethylthioethyl deoxyguanosine was formed from the reactions of hemi-sulfur mustard with deoxyguanosine or DNA (26-28).

The other CEES-modified deoxynucleosides and bases which are needed for identifying the radioactive peaks isolated from [^{14}C]-CEES-modified DNA can be obtained by reacting unlabeled CEES with the individual deoxynucleosides and bases as discussed under the various subheadings below.

O^6-Ethylthioethyl deoxyguanosine

When CEES was reacted with deoxyguanosine in aqueous solution at pH 7, a complex mixture of products was obtained. Although O^6-ethylthioethyl deoxyguanosine was a minor product at pH 7, it became a major product when the reaction was carried out in the presence of K_2CO_3. Deoxyguanosine (10 mg) was dissolved in 1 ml of DMSO in the presence of 20 mg of K_2CO_3; CEES (12 mg) was added and the mixture was allowed to react at 37°C for 48 hr. After DMSO was removed by repeatedly adding water and lyophilyzing to dryness, the residue was dissolved in 2 ml of water and separated by HPLC on a C_{18} reverse-phase column as described previously (24). O^6-Ethylthioethyl deoxyguanosine appeared as a symmetrical peak at 70 min. This material was collected and compared with the O^6-ethylthioethyl deoxyguanosine prepared from 6-chloro deoxyguanosine; it was identical in both ultraviolet spectra and chromatographic properties.

7-Ethylthioethyl deoxyguanosine and 7-ethylthioethyl guanine

When CEES was reacted with deoxyguanosine at pH 3.5, the mixture was enriched in both 7-ethylthioethyl deoxyguanosine and 7-ethylthioethyl guanine. Deoxyguanosine was dissolved at 1 mg/ml in 50 mM KH_2PO_4, pH 3.5, and reacted for 2 hr at 37°C with CEES at 1.4 mg/ml. HPLC separation of this reaction mixture revealed two major derivative peaks at 55 and 56.8 min in addition to large peaks of guanine and deoxyguanosine. These derivative peaks were collected and shown to be 7-ethylthioethyl deoxyguanosine and 7-ethylthioethyl guanine, respectively, as reported previously (24).

3-Ethylthioethyl adenine

Adenine, 1 mg/ml, was reacted with CEES, 1.6 mg/ml, in 25 mM sodium cacodylate buffer, pH 7, for 2.5 hr at 37°C. The pH was maintained at 7 by the addition of small amounts of 1 N NaOH. HPLC analysis of this reaction mixture showed the presence of several derivatives, one of which co-eluted with a major peak of radioactivity that was released from [^{14}C]-CEES-modified DNA.

This derivative peak was separated from unmodified adenine by low pressure column chromatography on a cation exchange column. Twenty milliliters of the CEES-adenine reaction mixture was applied to an SP-Sephadex C-25 column (1.5 x 18 cm) and eluted at 0.67 ml/min with 50 mM triethylammonium formate buffer, pH 4.5. The eluent was monitored for ultraviolet absorbance at 254 nm and 10-min fractions were collected. A large peak of ultraviolet-absorbing material appeared in fractions 5 through 20; this material had ultraviolet spectra and HPLC retention times identical with those of unmodified adenine. A smaller ultraviolet-absorbing peak appeared in fractions 23 through 31 which had an ultraviolet spectrum similar to that of 3-methyl adenine. Fractions containing this material were pooled, concentrated by lyophilization, and purified further by HPLC on a C_{18} reverse-phase column eluted with 10 mM triethylammonium formate buffer, pH 4.5, containing 10% acetonitrile. The volatile triethylammonium formate was removed by repeated lyophilizations, and the structure of the purified product, which appeared as a single peak in several chromatographic systems,

was determined to be 3-ethylthioethyl adenine by ultraviolet and mass spectrometry (24).

3-Ethylthioethyl deoxycytidine

Deoxycytidine, 1 mg/ml, was reacted with CEES, 4 mg/ml, in 50 mM sodium cacodylate buffer, pH 7.3, for 2 hr at 37°C while the pH was maintained by the addition of small quantities of 1 N NaOH. Again, HPLC analysis showed that this deoxynucleoside had been extensively modified by CEES.

The major derivative was separated from unreacted deoxycytidine on an SP-Sephadex C-25 cation exchange column (0.9 x 15 cm) eluted at 0.4 ml/min with 50 mM triethylammonium formate buffer, pH 4.5. The column was monitored at 254 nm, and one major derivative peak was observed which eluted just after the unmodified deoxycytidine. Fractions containing this derivative were pooled, concentrated by lyophilization, and purified further by HPLC on a C_{18} column eluted with 10 mM triethylammonium formate buffer, pH 6.5, containing 12% acetonitrile. After removal of the volatile buffer and acetonitrile by lyophilization, the product was determined to be 3-ethylthioethyl deoxycytidine by ultraviolet and mass spectrometry (24).

This derivative co-chromatographed with one of the peaks of radioactivity (peak 3) in HPLC profiles of enzyme-digested [^{14}C]-CEES-DNA shown in Figure 5 (see section on DNA modification). However, this derivative is stable to treatment at 70°C in 0.1 N HCl for 20 min, while radioactive peak 3 is not. Thus, we have concluded that 3-ethylthioethyl deoxycytidine is probably a very minor product when CEES reacts with DNA.

Surprisingly, 1,2-bis-ethylthioethane, $C_2H_5SC_2H_4SC_2H_5$, was found as a contaminant in the mass spectrum of 3-ethylthioethyl deoxycytidine. Since it is unlikely that 1,2-bis-ethylthioethane would have co-purified with 3-ethylthioethyl deoxycytidine, it must have been generated during mass spectrometry from the 3-ethylthioethyl deoxycytidine. This finding suggests that substituents attached to the deoxynucleosides by sulfur mustards may still retain some reactivity.

1-(3-Deoxycytidyl),2-(1-deoxyguanosinyl)ethane

This derivative was isolated from DNA treated with BCNU, and its structure was established as described in the Methods section and in the literature (12). Briefly, this derivative was obtained by separating an enzyme digest of BCNU-DNA on a cation exchange resin (12). 1-(3-Deoxycytidyl),2-(1-deoxyguanosinyl)ethane always elutes late from such a column and can be recognized by its HPLC retention time and its characteristic ultraviolet spectrum. Spectral properties, together with those of the other derivatives described in this section, are given in Table 2 below.

1-(3-Deoxycytidyl),2-(1-guanosinyl)ethane and 1-(3-Cytosinyl),2-(1-guanyl)ethane

1-(3-Deoxycytidyl),2-(1-guanosinyl)ethane, used as an intermediate in the synthesis of 1-(3-cytosinyl),2-(1-guanyl)ethane, was isolated from a reaction mixture of O^6fluoroethyl guanosine and deoxycytidine (see Methods section). This intermediate was separated from the reaction mixture by HPLC as shown in Figure 2. One quarter of the reaction mixture, 10 µl of dimethylsulfoxide which originally contained 1 mg of deoxycytidine and 0.5 mg of O^6-fluoroethyl guanosine, was injected on a C_{18} column (4.6 x 250 mm) and eluted as described in the figure legend.

Peaks 1 and 5 of this elution profile were identified as unreacted deoxycytidine and O^6-fluoroethyl guanosine, respectively, by comparison with known standards. Similarly, peak 2 was identified as 1-hydroxyethyl guanosine, in agreement with the literature report that O^6-fluoroethyl guanosine rearranges to this compound (29).

Peak 3 had ultraviolet spectra in acid, base, and neutral pH which corresponded to the spectra of a 1-substituted guanosine. The molecular weight of this derivative was 329 by fast atom bombardment mass spectrometry, establishing the substituent as a fluoroethyl group and resulting in the structural assignment of 1-fluoroethyl guanosine for peak 3.

Peak 4 had ultraviolet spectra in acid, base, and neutral pH identical with those of 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane. Its molecular weight by fast atom bombardment molecular spectroscopy corresponded to 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane. Considering the nature of the reaction mixture which led to the isolation of peak 4, and the fact that this material had the same ultraviolet spectrum as 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane and the correct molecular weight for 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane, it seemed very likely that the assignment of the structure, 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane, for peak 4 was correct.

Confirmation of this assignment came by subjecting material collected from peak 4 to acid hydrolysis. When this material was treated with 0.1 N HCl as described in the Methods section and separated by HPLC, the profile shown in Figure 3 was obtained. Three major peaks were evident: some remaining 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane (dC-rG) and two new products. Mass spectrometry revealed that material in the first peak (labeled C-G) had lost both of its sugars, while material in the second peak (labeled C-rG) had lost only the deoxyribose sugar and could be converted to C-G by further acid treatment.

Finally, we were able to show that the material in peak C-G corresponded to that obtained from the hydrolysis of authentic 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane. 1-(3-Deoxycytidyl),2-(1-deoxyguanosinyl)-ethane was subjected to acid hydrolysis and added to the hydrolysis products from 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane shown in Figure 3; the resulting HPLC profile is shown in Figure 4. This figure shows that the C-G peak has grown in size, demonstrating that the acid hydrolysis product of 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane and 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane is the same.

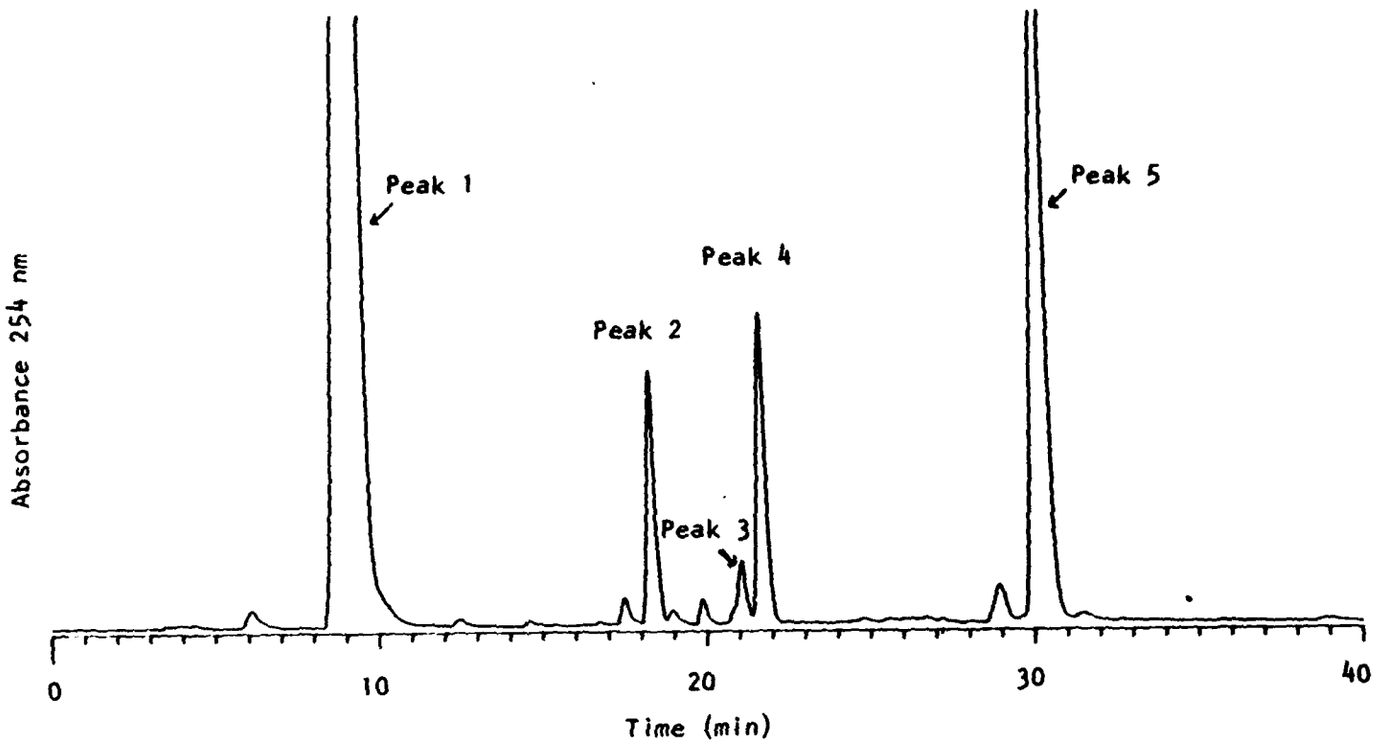

Figure 2. HPLC profile of deoxycytidine-O^6-fluoroethyl guanosine reaction mixture. An aliquot of the reaction mixture which originally contained 1 mg of deoxycytidine and 0.5 mg of O^6-fluoroethyl guanosine was separated on a C_{18} column (4.6 x 250 mm) eluted at a flow rate of 1 ml/min with a gradient of 1-10% acetonitrile in 50 mM K_2HPO_4, pH 6, over 20 min and then continued with 10% acetonitrile in the same buffer for 20 min.

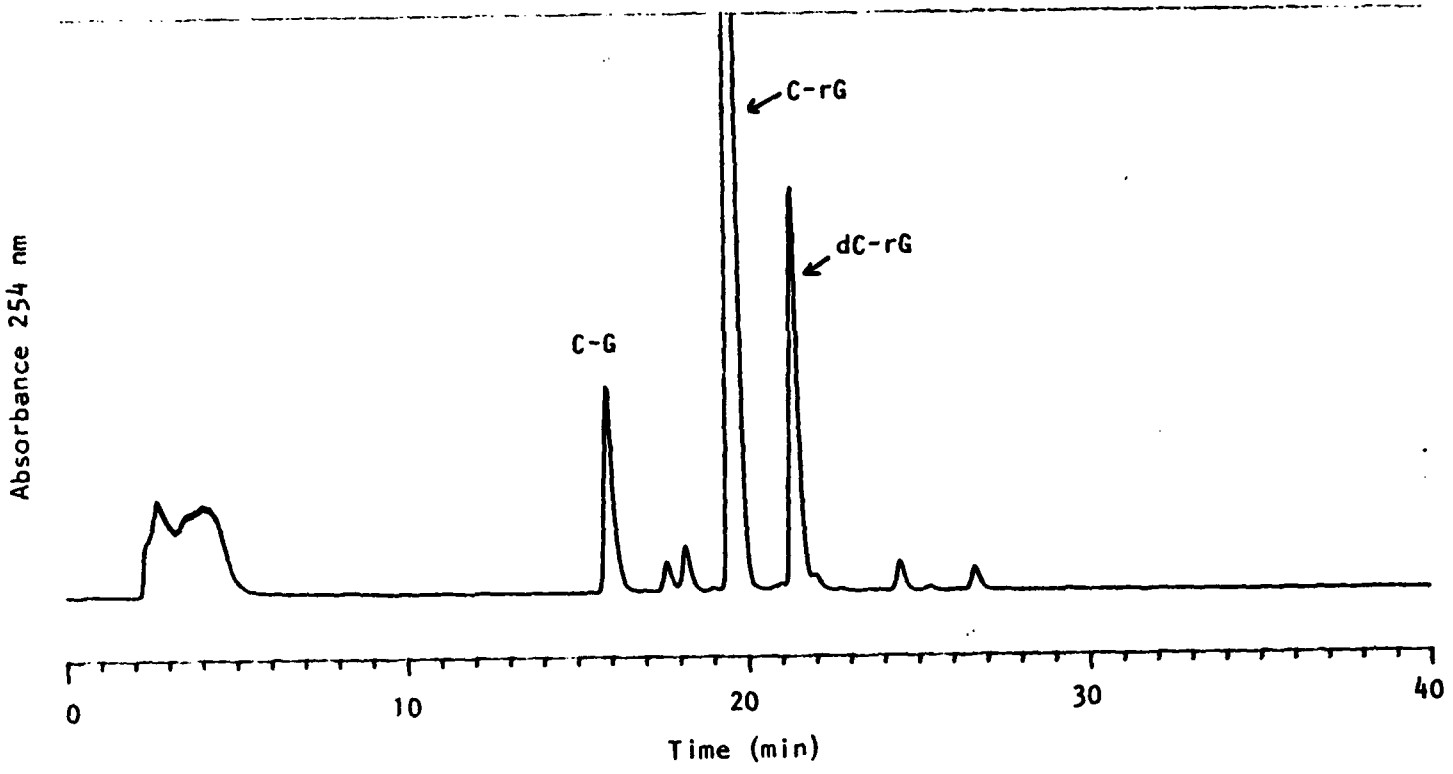

Figure 3. HPLC profile of acid-hydrolyzed 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane. Material from peak 4, Figure 2, was collected, subjected to mild acid hydrolysis, and separated on a C_{18} column as described in the legend to Figure 2. Peak identifications, as described in the text, are: C-G, 1-(3-cytosinyl),2-(1-guanyl)ethane; C-rG, 1-(3-cytosinyl),2-(1-guanosinyl)ethane; dC-rG, 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane.

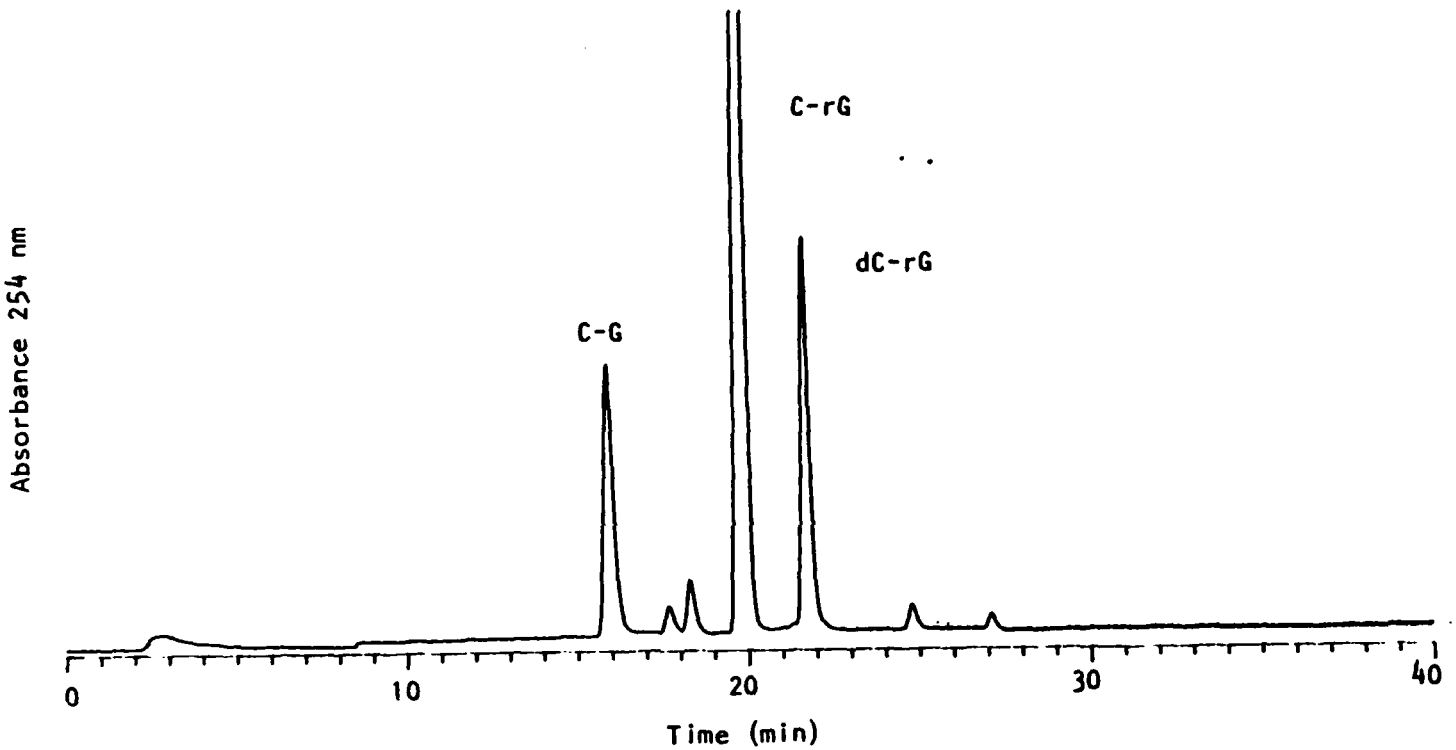

Figure 4. HPLC comparison of acid-hydrolyzed 1-(3-deoxycytidyl),2-(1-guanosinyl)ethane (dC-rG) and authentic 1-(3-cytosinyl),2-(1-guanyl)ethane (C-G). Authentic C-G was obtained by acid hydrolysis of 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane (dC-dG). When this material was added to the hydrolysis mixture shown in Figure 3 and rechromatographed under the same conditions, the C-G peak from dC-rG shown in Figure 3 co-chromatographed with authentic C-G.

Since the glycoside bond in purine nucleosides is generally more labile than in pyrimidine nucleosides and the bond in deoxyribose nucleosides is generally more labile than in ribose nucleosides, we were surprised to find that the more labile glycoside bond in 1-(3-deoxycytidyl),2-(1-guanosinyl)-ethane was that associated with the deoxyribose sugar. However, we compared the acid stability of 3-methyl deoxycytidine with 1-methyl guanosine and found that the latter was, indeed, more stable. The further implication of this observation is, of course, that 1-(3-cytosinyl),2-(1-guanyl)ethane (C-G) should be released from any DNA which contains this cross-link by mild acid hydrolysis. This conclusion was the basis for comparing the products released from acid-hydrolyzed [^{14}C]-CEES-DNA with marker C-G to see whether the [^{14}C]-CEES-DNA contained this particular cross-link as described below.

Summarizing this section, then, we have developed a relatively simple organic synthesis of the cross-link, 1-(3-cytosinyl),2-(1-guanyl)ethane, establishing its structure by a combination of ultraviolet and mass spectrometry and by comparison with the hydrolysis product of authentic 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane.

Ultraviolet Spectra of Modified Deoxynucleosides and Bases

In order to investigate the repair of sulfur mustard-induced DNA lesions, it is essential to have access to authentic HPLC markers of known derivatives, as explained at the beginning of this section. These derivatives are not available commercially, but the methods outlined above would permit an investigator to synthesize the modified deoxynucleosides and bases which are produced by chloroethyl ethyl sulfide.

The chemical identity of compounds synthesized in this way can be verified with a high degree of certainty by comparing their ultraviolet spectra with those given in Table 2 below. If necessary, further verification can be obtained by fast atom bombardment mass spectrometry.

Modification of DNA by [^{14}C]-CEES

DNA reacted with ^{14}C-labeled CEES at 29 µg/ml contained 6.3 nmol of ^{14}C-labeled derivatives per mg. To determine the distribution of this radioactivity, the DNA was digested as described above and separated by HPLC as shown in Figure 5. Seven distinct peaks of radioactivity were resolved; four of these co-eluted with known HPLC markers in this system, as shown in Table 3. Radioactive material isolated from each of these peaks also co-chromatographed with these markers in the four confirmatory HPLC systems described previously (27), thus verifying the assignments shown in Table 3.

The percent distribution of radioactivity among these peaks is shown in Table 3. In agreement with earlier reports (4,34,35), the 7 position of guanine was most readily alkylated; 7-ethylthioethyl deoxyguanosine and 7-ethylthioethyl guanine, together, accounted for 72% of the DNA modification. Also in agreement with earlier reports, the 3 position of adenine was the next most readily alkylated, and 3-ethylthioethyl adenine accounted for 11% of the total radioactivity. Both 7-ethylthioethyl deoxyguanosine and 3-ethylthioethyl deoxyadenosine depurinated readily under the digestion

conditions, so that these derivatives were represented primarily by the corresponding bases.

Table 2

Ultraviolet Spectra of Chloroethyl Ethyl Sulfide-modified DNA Bases and Deoxynucleosides

Compound	Acid		pH 7		Base	
	Max	Min	Max	Min	Max	Min
O^6-Ethylthioethyl deoxyguanosine	285 243	260 229	279 247	261 229	277 246	260 229
O^6-Hydroxyethylthio-ethyl deoxyguanosine	288 243	261	281 247	262	281 246	261
7-Ethylthioethyl deoxyguanosine	257 272(s)	231	254 283(s)	240 271	266	248
7-Ethylthioethyl guanine	250 272(s)	230	285	262	279	259
3-Ethylthioethyl adenine	274	236	275	243	273	246
3-Ethylthioethyl deoxycytidine	280	244	278	240	264	248
1-(3-Deoxycytidyl), 2-1-(deoxyguanosinyl) ethane	277	235	277	233	259	240
1-(3-Cytosinyl), 2-(1-guanyl)ethane	276	232	277	233	284	255

(s) shoulder.

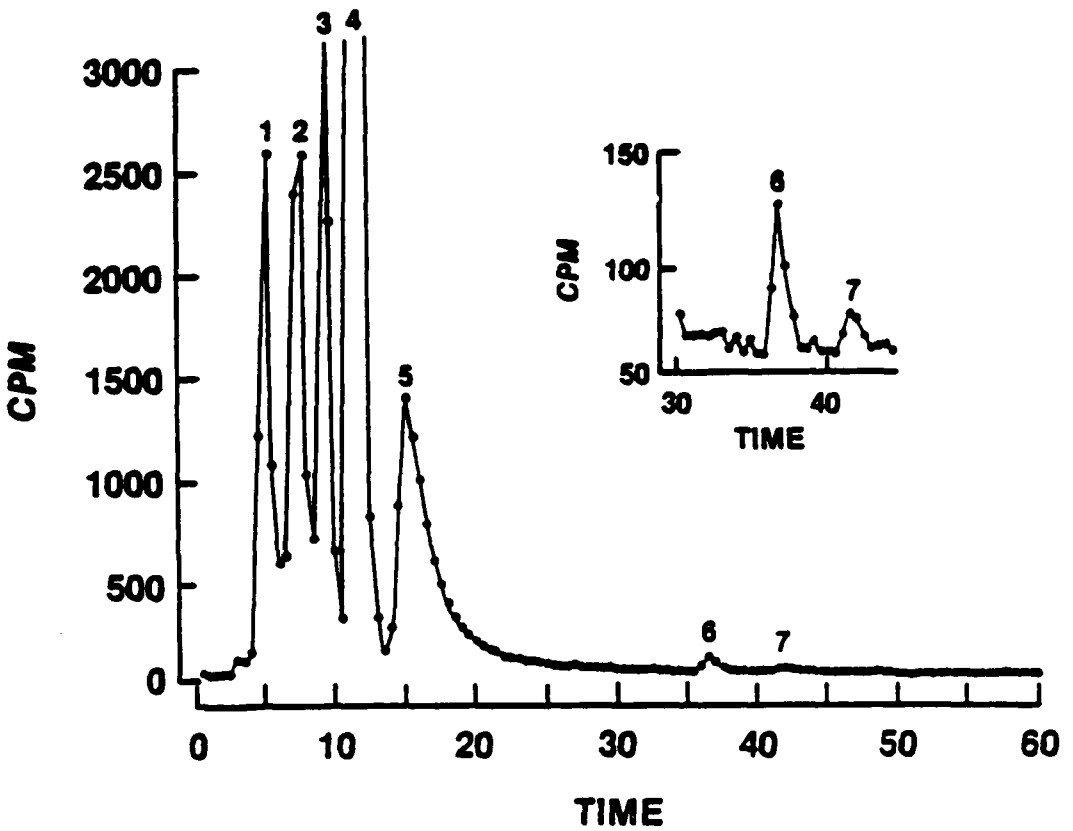

Figure 5. HPLC profile of digested [^{14}C]-CEES-modified DNA. DNA, modified as described in the text, was digested to the deoxynucleoside level and separated on a C_{18} column eluted at 1 ml/min with 25 mM KH_2PO_4, pH 4.5, containing 14% acetonitrile. 0.5-Min fractions were collected and counted for radioactivity. Peak identification is given in Table 3.

Table 3

Distribution of Radioactivity in
[^{14}C]-CEES-modified DNA

Peak No.	Identity	Percent of Total Radioactivity[a]	
1	Not yet identified	7.1	0.2
2	Not yet identified	9.0	0.3
3	7-Ethylthioethyl deoxyguanosine	9.1	0.4
4	7-Ethylthioethyl guanine	63.1	0.8
5	3-Ethylthioethyl adenine	11.4	0.2
6	Not yet identified	0.31	0.01
7	O^6-Ethylthioethyl deoxyguanosine	0.10	0.01

[a] Average of five independent determinations ± standard error.

O^6-Ethylthioethyl deoxyguanosine appeared as a small peak which eluted at approximately 42 min under these chromatographic conditions. This peak accounted for 0.1-0.3% of the total alkylation products as the overall level of substitution was varied by varying the reaction conditions.

Three derivative peaks, amounting to 16.4% of the total radioactivity, have not yet been identified. As described in the section on DNA cross-linking below, some of the radioactivity in peak 1 may be associated with 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane cross-links, but other possibilities include substituted pyrimidines. This suggestion would fit with the generally more polar nature of these derivatives.

The next step in identifying these unknown peaks would follow the procedure described under the cross-linking studies below. Marker compounds which might correspond to these unknowns would have to be synthesized and their identity established by co-chromatography.

Effect of Partially Purified Alkyl Transferase on [^{14}C]-CEES-modified DNA

Experiments to determine whether O^6-alkyl guanine-DNA alkyl transferase (alkyl transferase) from rat liver could repair O^6-ethylthioethyl deoxyguanosine in [^{14}C]-CEES-modified DNA were performed as shown in Figure 1. Incubations were performed in parallel with increasing amounts of alkyl transferase as shown in Table 4; control tubes contained no enzyme. Other controls utilizing bovine serum albumin or heat-inactivated alkyl transferase showed that nonspecific loss of radioactivity in the O^6-ethylthioethyl deoxyguanosine region could be prevented by the addition of 6 nmol of marker O^6-ethylthioethyl deoxyguanosine immediately following incubation with the repair enzyme.

As shown in Table 4, there was no evidence for repair of O^6-ethylthioethyl deoxyguanosine even when alkyl transferase was present in large excess. Averaging all of these experiments together, we find that the average percentage of O^6-ethylthioethyl deoxyguanosine in the controls is 0.10 ± 0.01 (SEM). In the enzyme-treated DNA's, the average percentage of O^6-ethylthioethyl deoxyguanosine is 0.11 ± 0.01. Although a small amount of repair could have escaped detection because O^6-ethylthioethyl deoxyguanosine is such a minor constituent in [^{14}C]-CEES-modified DNA, the conclusion seems inescapable that this enzyme has very little activity toward this substrate. As discussed below, this conclusion is also in agreement with recently published data which indicate that bulky alkyl groups like the ethylthioethyl group are not repaired by this enzyme.

In the course of these experiments, however, it was observed that peak 3 (7-ethylthioethyl deoxyguanosine) always diminished in size when substrate DNA was treated with alkyl transferase. These results are shown in Figure 6.

The most probable explanation for this loss of 7-ethylthioethyl deoxyguanosine is that some enzymatic activity in the partially purified rat liver alkyl transferase converts 7-ethylthioethyl deoxyguanosine to 7-ethylthioethyl guanine. Of course, 7-ethylthioethyl deoxyguanosine depurinates spontaneously to 7-ethylthioethyl guanine, as evidenced by the large peak of 7-ethylthioethyl guanine which appears in the enzyme digest of control tubes which have not been exposed to partially purified rat liver alkyl transferase (see Figure 5). However, the fact that the 7-ethylthioethyl deoxyguanosine peak diminishes further in a manner dependent on the amount of alkyl transferase added, as shown in Figure 6, indicates that some activity in the rat liver extract is accelerating this reaction.

Experiments which would detect the direct release of 7-ethylthioethyl guanine into the supernatant have been inconclusive because crude rat liver extracts which might contain a 7-alkyl guanine DNA glycosylase also contain endonucleases which release radioactivity into the supernatant in a nonspecific way. Further experiments to demonstrate the presence of 7-alkyl guanine DNA glycosylase should be performed with a double labeled substrate as described in the Methods section.

Table 4

Effect of Partially Purified Alkyl Transferase on O^6-Ethylthioethyl Deoxyguanosine (O^6ETEdGR) in [^{14}C]-CEES-modified DNA

Experiment No.	Enzyme added[a]	cpm O^6ETEdGR[b]	Total cpm eluted	Percent O^6ETEdGR	Pmol of O^6ETEdGR/mg DNA
	1.6	86.4	67,650	0.13	8.2
2	0	42.0	72,883	0.06	3.8
	4.9	90.8	72,890	0.12	7.6
3	0	44.2	66,106	0.07	4.4
	9.9	38.4	53,086	0.07	4.4
4	0	87.4	70,392	0.12	7.6
	11.5	57.7	67,692	0.09	5.7
5	0	88.2	72,430	0.12	7.6
	19.8	63.1	48,602	0.13	8.2

[a] Units of enzyme/pmol of O^6ETEdGR.

[b] Counts per minute (peak - background).

Cross-linking of DNA by CEES

Evidence for Cross-linking: Ethidium Bromide Fluorescence Method

Data obtained by the ethidium bromide method provided clear-cut evidence that the monofunctional sulfur mustard, chloroethyl ethyl sulfide, does, in fact, cross-link DNA. Table 5, below, compares the fluorescence after denaturation of a control (unmodified DNA), two DNA's treated with nitrogen mustard (HN2), and two DNA's treated with CEES. Each of these samples had a fluorescence of approximately 650 units before denaturation. After denaturation, thi- was reduced to 36 units for the unmodified DNA, a reading which represents scattered light and other sources of background current. The positive controls, DNA's cross-linked by 4 μM and 20 μM concentrations of nitrogen mustard, respectively, gave readings of 70 and 149 fluorescence units after denaturation. These levels indicate significant intercalation of ethidium bromide and, accordingly, considerable renaturation of this cross-linked DNA to double helical form.

DNA's treated with chloroethyl ethyl sulfide also showed evidence of cross-linking in a dose-dependent way. As anticipated, however, much higher concentrations of this single armed mustard were necessary to produce

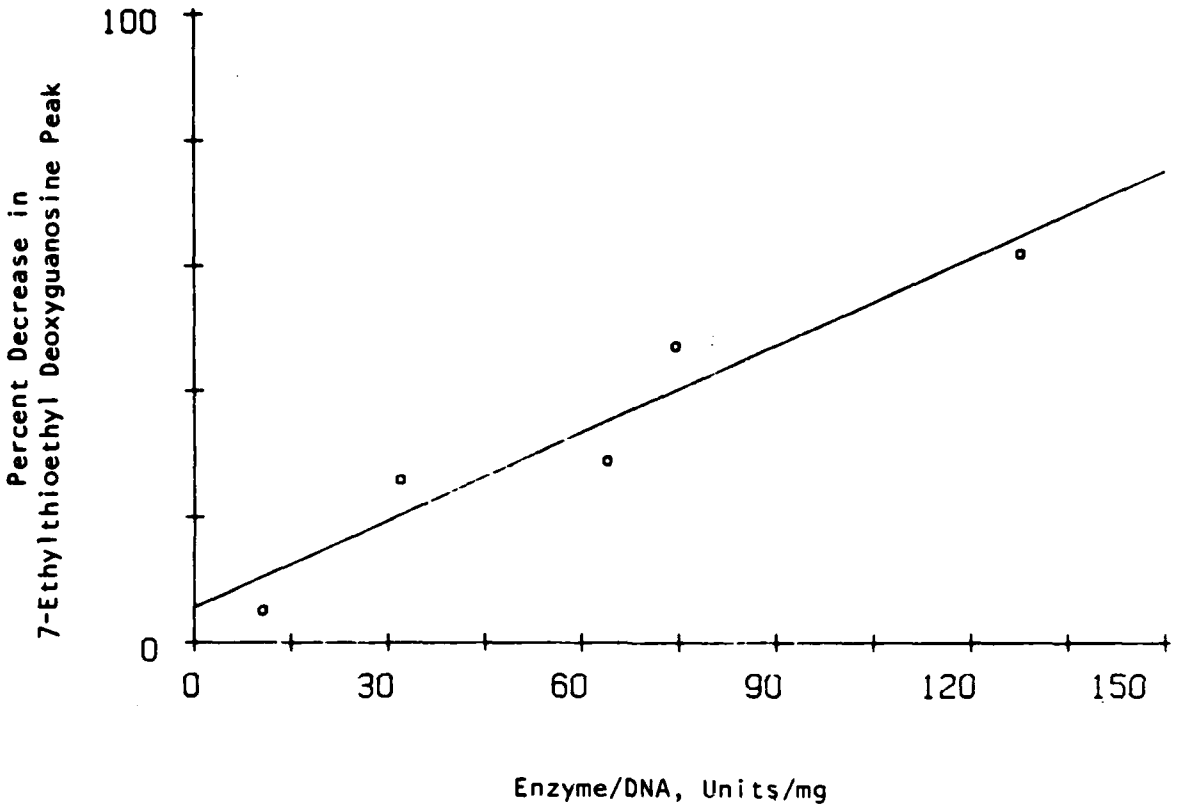

Figure 6. Effect of partially purified alkyl transferase on 7-ethylthioethyl deoxyguanosine in [^{14}C]-CEES-modified DNA. Substrate DNA was incubated with partially purified rat liver alkyl transferase using the protocol shown in Figure 1. The percent decrease in the 7-ethylthioethyl deoxyguanosine peak (peak 3 in Figure 5) is plotted against the alkyl transferase/DNA ratio, expressed in units of enzyme/mg of DNA.

measurable cross-linking. Chloroethyl ethyl sulfide concentrations of 234 μM and 830 μM produced 44 and 50 units of fluorescence intensity after denaturation, respectively. No attempt was made to determine exactly how many cross-links per molecule of DNA this represents, however, because the primary purpose of these experiments was to demonstrate that this single armed mustard cross-links DNA. Further efforts were directed toward determining whether the cross-links represent formation of 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane within DNA.

Table 5

DNA Cross-linking by Chloroethyl Ethyl Sulfide as Determined by Fluorescence Method

DNA No.	Treatment	Concentration of agent (μM)	DNA alkylation (nmol/mg)	Fluorescence[a]	
				Before denaturation	After denaturation
A218	Unmodified	0	0	640	36
S114A	HN2	4	[b]	680	70
S114B	HN2	20	[c]	630	149
A527	CEES	234	6.3	680	44
A782	CEES	830	27.1	650	50

[a] Fluorescence intensity is in arbitrary units as measured on a Perkin-Elmer spectrofluorometer, sensitivity = 30.

[b] DNA was reacted with 4 μM HN2 at 37°C under conditions which should cross-link 80% of the DNA (36).

[c] DNA was reacted with 20 μM HN2 at 37°C under conditions which should cross-link 100% of the DNA (36).

Evidence for 1-(3-Deoxycytidyl),2-(1-deoxyguanosinyl)ethane Cross-links in CEES-modified DNA

The relatively low level of cross-links found in DNA's treated with monofunctional sulfur mustards makes it difficult to isolate enough material from DNA to establish the structure of the cross-link directly. Therefore, an alternate approach was used: DNA was treated with [^{14}C]-CEES, digested to the deoxynucleoside level, and the radioactive derivatives in this digest were compared by HPLC to authentic 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane to see whether this particular cross-link was present.

DNA was treated with [^{14}C]-CEES and digested to the deoxynucleoside level as described in the Methods section. When this digest was separated by HPLC under the conditions shown in Figure 5, marker 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane eluted at 4.1 min, corresponding to one of the unknown derivatives in this profile. Additional separations were then performed at lower concentrations of acetonitrile to improve the resolution in this region.

At each concentration of acetonitrile, a small peak of labeled material co-eluted with marker 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)-ethane, a result which is fully consistent with the presence of this cross-link in [^{14}C]-CEES-modified DNA. A typical elution profile is shown in Figure 7.

Further confirmation of the presence of this cross-link was obtained by treating the enzyme digest of [^{14}C]-CEES-modified DNA with acid to remove the sugars which are part of the 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane structure. This acid-treated digest was then separated by HPLC together with 1-(3-cytosinyl),2-(1-guanyl)ethane marker to determine whether any radioactivity was found in this region. Again, a small peak of radioactivity co-eluted with the marker.

If this co-eluting material is really 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane or in the case of the acid-treated digest, 1-(3-cytosinyl),2-(1-guanyl)ethane, the percentage of radioactivity associated with this cross-link should be the same regardless of the HPLC elution system. These percentages were calculated for each chromatographic system and are shown in Table 6. Values for the percent of radioactivity associated with marker average 0.5%, and the agreement among the different determinations is quite good considering the different chromatographic conditions and hydrolysis methods used. We conclude, therefore, that 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane may be present in DNA treated with CEES.

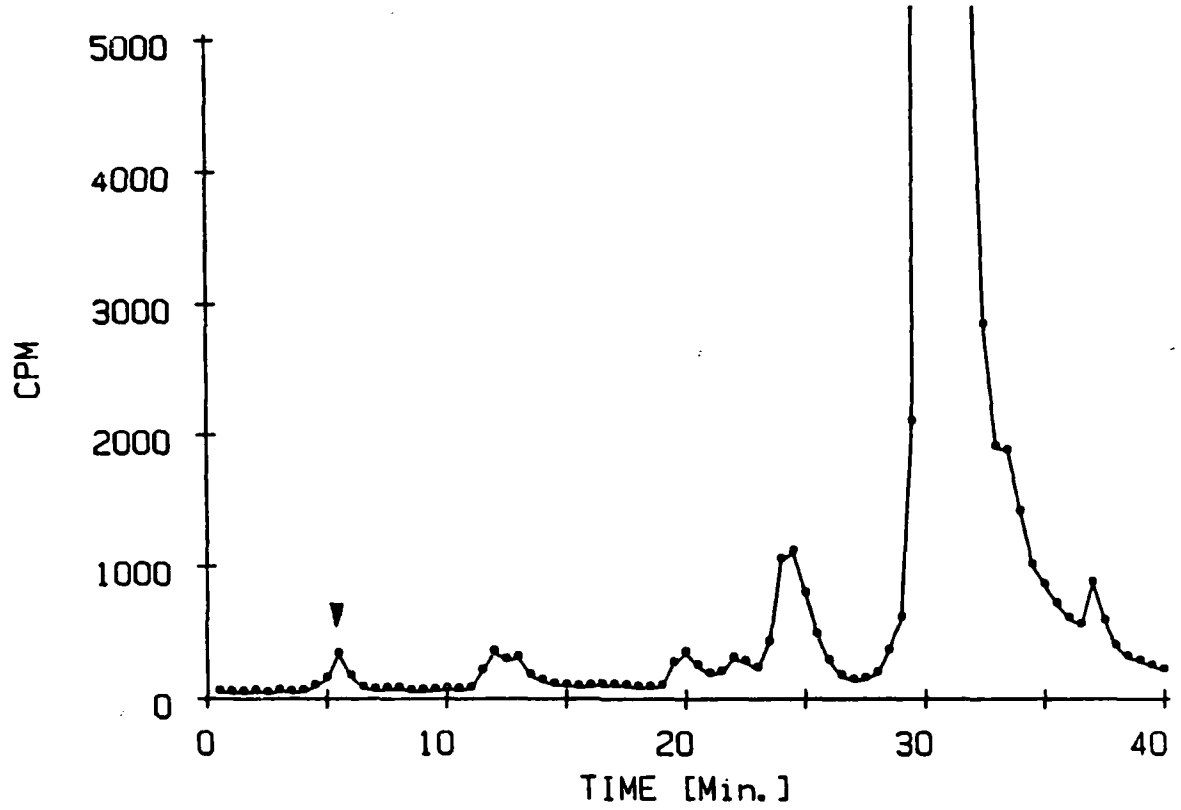

Figure 7. HPLC comparison of digested [^{14}C]-CEES-modified DNA and authentic 1-(3-cytosinyl),2-(1-guanyl)ethane. [^{14}C]-CEES-modified DNA was digested to the deoxynucleoside level and then treated with dilute HCl. Marker 1-(3-cytosinyl),2-(1-guanyl)ethane was added, and the mixture was separated by HPLC as described in Footnote d of Table 6; 0.5-min fractions were collected and counted. A small peak of radioactivity co-eluted with the marker which appeared at the position indicated by the arrow.

Table 6

Chromatographic Evidence for
1-(3-Deoxycytidyl),2-(1-deoxyguanosinyl)ethane
(dC-dG) Cross-links in [^{14}C]-CEES-modified DNA

Experiment Number	Hydrolysis Method	Expected Cross-link	HPLC Conditions	Percent of cpm's Co-eluting with Marker
559	Enzymatic	dC-dG	(a)	0.6
664	Enzymatic	dC-dG	(b)	0.6
877	Enzymatic	dC-dG	(c)	0.3
1043	Enzymatic + HCl treatment	C-G	(d)	0.7

(a) Isocratic elution from a C_{18} column at 1 ml/min with 25 mM KH_2PO_4, pH 4.5, containing 14% acetonitrile.

(b) Isocratic elution from a C_{18} column at 1 ml/min with 25 mM KH_2PO_4, pH 4.5, containing 10% acetonitrile.

(c) Isocratic elution from a C_{18} column at 1 ml/min with 50 mM KH_2PO_4, pH 4.5, containing 8% acetonitrile for 15 min and then with an 8-25% gradient of acetonitrile in the same buffer over a 25-min period.

(d) Gradient elution from a C_{18} column at 1 ml/min: 1-10% acetonitrile in 50 mM K_2HPO_4, pH 6, over a 40-min period; then, 10-75% acetonitrile in the same buffer over a 10-min period, continuing with 75% acetonitrile in the same buffer for 10 min.

DISCUSSION

Modification of DNA by Chloroethyl Ethyl Sulfide

The demonstration that sulfur mustards alkylate the O^6-position of guanine in DNA is very significant. This reaction had been proposed earlier to explain some of the biological effects of the sulfur mustards (7), but had not been demonstrated previously. The availability of newer laboratory techniques has made it possible to demonstrate this modification at both the deoxynucleoside and DNA levels.

Recent investigations in chemical carcinogenesis (15) have emphasized the importance of minor modifications of DNA. Thus, even though the bulk of DNA modification by the sulfur mustards is at the 7 position of guanine and the 3 position of adenine, alkylation of the O^6-position of guanine is probably very important. This modification is known to be a miscoding lesion in DNA (37,38) and a likely cause of cancer.

There is an additional reason why alkylation of the O^6-position of guanine could be important: substitution at this position leads to DNA cross-linking by some antitumor agents (12) and a similar reaction could occur with the sulfur mustards. This mechanism is shown in Figure 8 and involves an initial substitution of the O^6-position of guanine; in the case of CEES, the first step would be formation of O^6-ethylthioethyl guanine. Loss of the thioethyl group would lead to the formation of the five-membered ethano ring shown in Step 3 of the figure. DNA cross-linking would then follow as it does with the haloethyl nitrosoureas.

An interesting feature of this cross-linking mechanism is that it can be interrupted by alkyl transferase if the substituent at the O^6-position of guanine is a substrate for this enzyme. This is evidently a major cause of resistance to the cytotoxic effects of the haloethyl nitrosoureas and was one of the reasons for examining the repair of O^6-ethylthioethyl deoxyguanosine as described below.

The data obtained with the ethidium bromide method which are shown in Table 5 demonstrate that the one-armed sulfur mustard, chloroethyl ethyl sulfide, does indeed form cross-links in DNA. Furthermore, the chromatographic evidence presented in Table 6 is fully consistent with the formation of the cross-link shown in Figure 8. More studies would have to be performed to prove this point with certainty, but the presence of radioactivity which co-elutes with the cross-link marker under different chromatographic conditions strongly suggests that this lesion is present in [^{14}C]-CEES-modified DNA.

The possibility that alkylation of the O^6-position of guanine is involved in cross-link formation clearly adds importance to this DNA modification. Although it appears that alkyl transferase cannot repair this lesion, other repair mechanisms probably can. If so, this would provide protection against the mispairing associated with O^6-alkyl guanine and against cross-link formation as well.

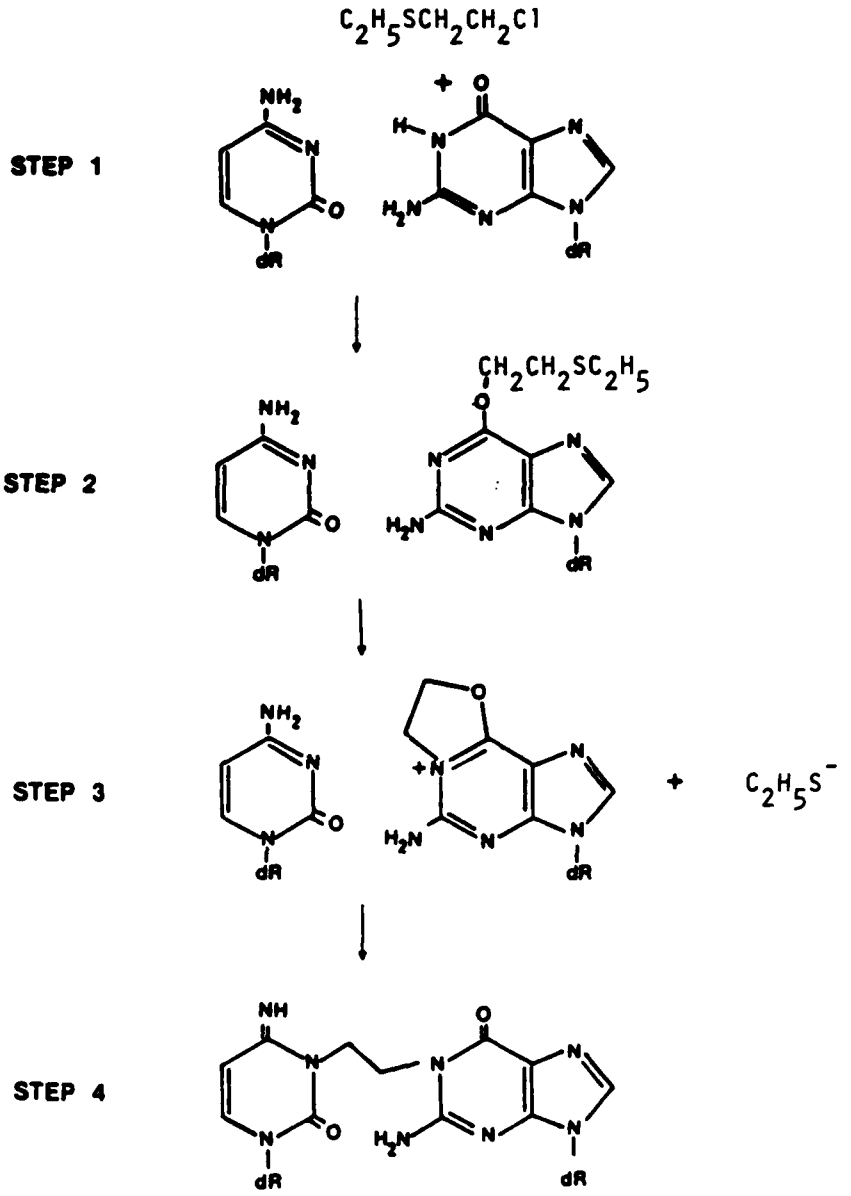

Figure 8. Proposed mechanism for the formation of a 1-(3-deoxycytidyl),2-(1-deoxyguanosinyl)ethane cross-link in DNA by a sulfur mustard. Initial attachment of the sulfur mustard to the O^6-position of guanine is followed by rearrangement and cross-linking with a deoxycytidine moiety in the opposite DNA strand.

Another important point emerges from these studies. Although the major sites of DNA modification by the sulfur mustards have been correctly identified as the N-7 position of guanine and the N-3 position of adenine (3-5), three of the derivative peaks in Figure 5 totaling 16.4% of the total alkylation have not yet been identified. Given our current understanding of the importance of minor sites of DNA modification, some of these remaining derivatives could be very important.

Repair of Sulfur Mustard-induced DNA Damage

The repair experiments reported above were designed primarily to investigate the effectiveness of alkyl transferase in repairing O^6-ethylthioethyl deoxyguanosine in [^{14}C]-CEES-modified DNA. The results given in Table 4 indicate that the enzyme is either totally inactive toward this substrate or has very low activity.

These results are in good agreement with the recent report by Morimoto et al. (22) that both E. coli and rat liver O^6-alkylguanine-DNA alkyl transferase show less activity for larger, bulkier alkyl groups than they do for methyl and ethyl groups. Although it would be impossible to rule out some very slow repair of O^6-ethylthioethyl guanine by this enzyme, it is clear that alkyl transferase would be very inefficient in repairing this lesion. Bulky DNA adducts are, however, often repaired by the UVR excision endonuclease so the cell is probably not entirely without defenses to the damaging effects of the sulfur mustards.

In spite of the fact that the repair experiments reported here were designed to investigate the effect of an alkyl transferase, they do provide some direct evidence for DNA repair by another mechanism. As indicated by the data presented in Figure 6, the amount of 7-ethylthioethyl deoxyguanosine in the substrate decreases during incubation with alkyl transferase in an enzyme-dependent manner. This would seem to indicate that a 7-alkyl guanine DNA glycosylase recognizes 7-ethylthioethyl deoxyguanosine in DNA and cleaves the glycoside bond releasing 7-ethylthioethyl guanine. As described in the Methods section, a better experimental protocol for detecting this enzyme is to examine the supernatant for labeled 7-ethylthioethyl guanine released from a DNA which contains [^{3}H]-thymidine to control for endonuclease action. It would be important to investigate this lead because a 7-alkyl guanine DNA glycosylase which recognizes sulfur mustard-induced 7-alkylation of guanine would probably be capable of repairing the usual sulfur mustard-induced cross-links in DNA.

Thus, although these studies have shown that alkyl transferase is probably not effective in repairing sulfur mustard-induced DNA damage, they have provided the methodology for examining other kinds of DNA repair. Furthermore, they have provided preliminary evidence for a glycosylase activity which would repair the major sulfur mustard-induced cross-link in DNA, a cross-link which involves the linkage of two neighboring guanines by a bridge attached to their N-7 positions.

SIGNIFICANCE

The relationship of DNA modification and repair to the toxicities seen in mustard gas poisoning is best presented in the context of cancer chemotherapy in which exposure to agents which have systemic effects like those of the mustards is part of the treatment of malignant disease. Although the effects of the sulfur mustards on the skin are unique and extremely important, the systemic effects which appear approximately a week after exposure are very difficult to manage and are very similar to those seen in cancer chemotherapy.

This semiacute toxicity is related to bone marrow depression in both cases, and the usual medical treatment consists of supportive care to deal with the infections and bleeding which often follow. The practical difference is that in cancer chemotherapy the administered dose is controlled and known. Recently, however, it has been realized that there are variations in absorption and metabolism of the chemotherapeutic agents, and the extent of DNA modification has been used to judge the response effective dose received (39). This model could be put to immediate use in judging the seriousness of systemic exposure to the sulfur mustards if methods were developed to measure the amount of DNA damage in the cells of exposed individuals. Practically, these measurements are usually made on circulating lymphocytes.

Accordingly, a detailed knowledge of the relationship between exposure and DNA damage would be of great assistance in determining the seriousness of exposure to the mustards. The methodology and results obtained in this report provide the approach to this problem, but the studies should be extended to bis-chloroethyl sulfide (mustard gas) itself.

The knowledge gained in the studies reported here helps to explain some of the biological effects of the mustards. The finding that the O^6-position of guanine is modified by these agents is the probable explanation for their mutagenic and carcinogenic effects. By developing the methodology necessary to study the repair of this lesion, it is now possible to determine what cellular mechanisms provide protection against this and other DNA modifications.

The finding that alkyl transferase is not effective in repairing this lesion is, of course, disappointing. However, it is now clear that the sulfur mustards cause a variety of DNA modifications and that many different repair processes operate to protect cells from the consequences of this damage. Again, borrowing from knowledge gained in the area of cancer chemotherapy, we know that mammalian cells can develop resistance to the effects of alkylating agents (17). Further studies of DNA repair will determine which repair mechanisms allow cells to cope with moderate amounts of sulfur mustard-induced damage and suggest methods of increasing these defense mechanisms.

RECOMMENDATIONS

The author would like to recommend that the studies of DNA modification and repair performed under this contract be continued with sulfur mustard itself. Since the semiacute toxicity of these agents, especially the bone marrow depression, is apt to be a very serious problem in combination with the initial skin lesions, it would be important to determine which DNA modifications are responsible for these problems and to develop methods of assaying this damage. Recent advances in cancer chemotherapy provide a model for establishing the relationship between damage to circulating lymphocytes and systemic toxicity, and this approach should be successful in evaluating individuals exposed to the sulfur mustards and similar agents.

Recent data, also from the field of cancer chemotherapy, have shown that cells can develop resistance to the effects of alkylating agents, presumably by increasing their capacity to repair DNA. Accordingly, every effort should be made to identify these protective mechanisms and to determine whether they can be induced to provide increased protection to the toxic effects of the sulfur mustards.

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APPENDIX

List of Publications Supported by This Contract

Direct Synthesis of the DNA Crosslink, 1-(3-Cytosinyl),2-(1-guanyl) ethane. D. B. Ludlum, M. C. Kirk, and J. MacFarland. In preparation.

Formation of O^6-Ethylthioethylguanine in DNA by Reaction with the Sulfur Mustard, Chloroethyl Ethyl Sulfide, and Its Apparent Lack of Repair by O^6-Alkylguanine-DNA Alkyltransferase. D. B. Ludlum, S. Kent, and J. R. Mehta. Carcinogenesis 7, 1037-1042 (1986).

DNA Modification by Sulfur Mustards and Nitrosoureas and Repair of These Lesions. D. B. Ludlum and B. Papirmeister. In: Mechanisms of DNA Damage and Repair: Implications for Carcinogenesis and Risk Assessment. M. G. Simic, L. Grossman, and A. Upton (eds.), Plenum, New York, 1986, pp. 119-126.

Formation of O^6-Ethylthioethyl Deoxyguanosine from the Reaction of Chloroethyl Ethyl Sulfide with Deoxyguanosine. D. B. Ludlum, W. P. Tong, J. R. Mehta, M. C. Kirk, and B. Papirmeister. Cancer Res. 44, 5698-5701 (1984).

List of Personnel Receiving Contract Support

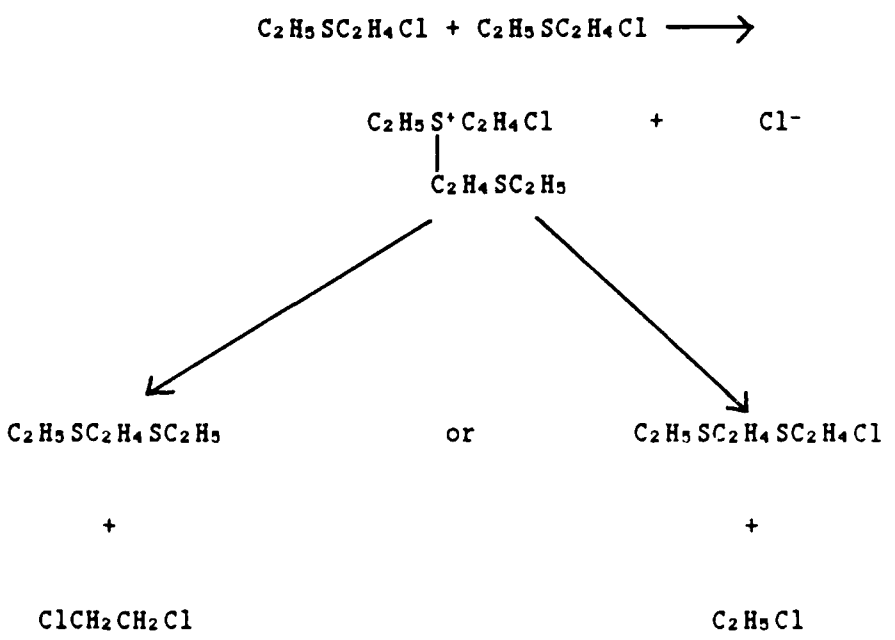
Name	Position
David B. Ludlum, Ph.D., M.D.	Principal Investigator
Yvette Habraken, Ph.D.	Research Associate
Sally Kent, B.A.	Research Assistant
Joseph MacFarland, B.Sc.	Research Assistant
Robert Colinas, B.Sc.	Research Assistant
Andrea Altwicker, B.Sc.	Research Assistant
Santas Seitz, M.S.	Research Assistant
Beth Coulter, B.A.	Research Assistant

Polymerization of Chloroethyl Ethyl Sulfide

Chloroethyl ethyl sulfide was noted to lose its reactivity after storage at room temperature or in a freezer. This was evidenced by a decrease in the amount of modified deoxynucleosides formed in reactions between deoxynucleosides and CEES and by a decrease in the amount of acid released during these reactions. No differences were noted in the chemical identity of the modified deoxynucleosides which were produced with aged CEES, however.

To investigate this loss of reactivity, CEES was analyzed by combined gas chromatography/mass spectrometry. In this procedure, the components of the mixture were separated on a chromatographic column and analyzed by mass spectrometry. The data obtained from this analysis are fully documented in a previous report (24).

Two products were identified in slightly aged CEES, which are indicative of polymerization: 1,2-bis-ethylthioethane and 1-ethylthio,2-chloroethylthioethane. The presence of these compounds and the loss of reactivity of CEES with time support the following polymerization scheme:



Note that polymerization to 1-ethylthio,2-chloroethylthioethane only partially inactivates CEES because this compound is still a sulfur mustard.

Although not immediately relevant to our studies of CEES, it is clear that a similar polymerization reaction would occur with sulfur mustard. In this case, all of the sulfur-containing products would be sulfur mustards, but they would have higher molecular weights and lower diffusion constants than the original bis-chloroethyl sulfide.

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